REMARKS

The April 21, 2006 Official Action and the references cited therein have been carefully reviewed. In view of the amendments submitted herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, it is noted that a shortened statutory response period of three (3) months was set forth in the April 21, 2006 Official Action. Therefore, the initial due date for response is July 21, 2006.

As another preliminary matter, the Examiner contends that claims 53 and 56-58 are not fully supported by U.S. Patent Application 09/851,327. As such, it is the Examiner's position that the instant application is a continuation-in-part of the '327 application and not a divisional application as recited in the priority claim. Applicants respectfully disagree with the Examiner's position. However, in the interest of expediting prosecution of the instant application, Applicants have amended claims 56-58. Support for the amendments to claims 56-58 can be found, for example, at page 16, lines 12-13 and page 18, lines 11-17. With regard to claim 53, Applicants respectfully submit that the disclosure cited by the Examiner at page 4 of the instant Official Action provides clear support for the method of claim 53. Indeed, at page 23, line 18 through page 24, line 10, the specification teaches that Tenascin C can upregulate expression of TB4 thereby enhancing the enhancing the cytoskeletal permissiveness of the cell for transfection. Further, it is disclosed that Tenascin C alters vascular smooth muscle cell shape and that denatured collagen or a peptide thereof induces Tenascin C. The instant specification also discloses the increased transfection of vascular smooth muscle cells with denatured collagen (see Example 1). Clearly, nothing more is needed to support the instant claim.

At page 6 of the instant Official Action, the Examiner contends that the previously submitted declaration is

defective for allegedly failing to identify that the instant application was amended on September 5, 2003. Applicants respectfully disagree and submit that no new declaration is required in view of the clear support present in the specification.

The Examiner has objected to the specification on the following two grounds. First, the Examiner has requested the priority claim be amended to indicate that the '327 application has issued as U.S. Patent 6,919,208. Applicants have made the appropriate amendments to the priority claim, thereby overcoming the instant objection. Second, the Examiner has objected to the specification under 37 CFR §1.75 (d)(1) for allegedly failing to provide proper antecedent basis for the claimed subject matter. Specifically, the Examiner contends that the specification fails to provide support for claims 53 and 56-58 as presented in the Preliminary Amendment. As stated hereinabove, Applicants respectfully disagree with the position taken by the Examiner. As such, the instant objection has been overcome as the instant specification fully supports the instantly claimed invention.

The Examiner has objected to claims 34, 35, 41-46, 52-60, and 66 for allegedly encompassing non-elected inventions. Applicants have amended claims 34, 45, and 59, from which the other claims depend, to recite that the agent is denatured collagen or a peptide of denatured collagen, thereby overcoming the instant objection.

The Examiner has also rejected claims 34, 35, 40-44, 57, and 58 for allegedly failing to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph on two grounds.

Claim 65 has been rejected under 35 U.S.C. §112, second paragraph for alleged indefiniteness. Specifically, claim 65 recites "the method of claim 60," but claim 60 is drawn to a kit. Applicants have amended claim 65 to recite the kit of claim 60, thereby overcoming the instant rejection.

The Examiner has rejected claims 34, 35, 40-46, 51,

53, 55, 59, 60, 65, and 66 under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 5,849,902.

Claims 34, 35, 40, 41, 44-46, 51-60, 65, and 66 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 5,652,225.

Lastly, claims 34, 35, 40, 41, 44-46, 51-54, 56, 59, 60, 65, and 66 have been rejected under 35 U.S.C. §§102(a) and 102(e) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 6,025,337.

The foregoing objections and rejections constitute all of the grounds set forth in the April 21, 2006 Official Action for refusing the present application.

In accordance with instant amendment, claims 67-70 have been added. Support for claim 67 can be found throughout the specification including, for example, in original claim 1 and Example 1. Support for new claims 68-70 can be found, for example, at page 28, lines 10-11.

No new matter has been introduced into this application by reason of any of the amendments presented herewith.

In view of the present amendment and the reasons set forth in this response, Applicants respectfully submit that the objections to the specification; the objection to the claims; the 35 U.S.C. §112, first paragraph rejections of claims 34, 35, 40-44, 57, and 58; the 35 U.S.C. §112, second paragraph rejection of claim 65; and the 35 U.S.C. §102/103 rejections of claims 34, 35, 40-46, 51-60, 65, and 66, as set forth in the April 21, 2006 Official Action, cannot be maintained. These grounds of objection and rejection are, therefore, respectfully traversed.

CLAIMS 34, 35, 40-44, 57, AND 58, AS AMENDED, SATISFY THE ENABLEMENT REQUIREMENT OF 35 U.S.C. §112, FIRST PARAGRAPH

The Examiner has rejected claims 34, 35, 40-44, 57, and 58 for allegedly failing to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph on the following two grounds.

First, with regard to claims 34, 35, and 40-44, it is the Examiner's position that while the specification is enabling for enhancing transfection of cultured cells in the presence of tenascin C, denatured collagen, or cytochalasin D, the specification allegedly does not provide enablement for any other embodiments embraced by the claims. As stated hereinabove, Applicants have amended the claims to recite that the methods for increasing transfection have been amended to recite that denatured collagen or a peptide of denatured collagen has been provided to the cell. Accordingly, Applicants submit that the instant enablement rejection has been overcome.

Second, the Examiner contends that claims 57 and 58 fail to comply with the enablement requirement because the claimed compositions are "taught in the specification only as being for *in vivo* use." The Examiner also contends that "in view of the state of the art, the high unpredictability of in vivo transfection, the lack of relevant working examples for *in vivo* transfection, the lack of guidance commensurate in scope with the claims, and the amount and nature of the experimentation required to develop those embodiments..., it would require undue experimentation to practice the invention commensurate in scope with the claims."

Applicants respectfully disagree with the Examiner's position and submit herewith Perlstein et al. (Gene Ther. (2003) 10:1420-1428). Perlstein et al. clearly teach that stents comprising nucleic acid molecules and denatured collagen increase the transfection of the nucleic acid molecule *in vivo* (see page 1423 and Figure 6). Notably, at page 18 of the instant specification, it is disclosed that

agents capable of enhancing cytoskeletal permissiveness of the cell for transfection, e.g., denatured collagen, can be administered via stents with the nucleic acid molecule to be transfected. Furthermore, the instant specification discloses at page 16 that the denatured collagen and the nucleic acid molecule can be contained with a polymeric carrier such as a controlled release film. Accordingly, Applicants submit, particularly in view of Perlstein et al., that the recited compositions of claims 57 and 58 are clearly enabled.

In view of all of the foregoing, Applicants respectfully submit that the enablement rejections of claims 34, 35, 40-44, 57, and 58 cannot be reasonably maintained and request their withdrawal.

CLAIMS 34, 35, 40-46, 51-60, 65, AND 66, AS AMENDED, ARE NOT ANTICIPATED OR RENDERED OBVIOUS BY THE CITED REFERENCES

The Examiner has rejected claims 34, 35, 40-46, 51, 53, 55, 59, 60, 65, and 66 under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 5,849,902. Claims 34, 35, 40, 41, 44-46, 51-60, 65, and 66 have also been rejected under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 5,652,225. Claims 34, 35, 40, 41, 44-46, 51-54, 56, 59, 60, 65, and 66 have been rejected under 35 U.S.C. §§102(a) and 102(e) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent 6,025,337.

Applicants respectfully disagree with the Examiner's position. It is a well-settled premise in patent law that "silence in a reference is not a proper substitute for adequate disclosure of facts from which a conclusion of obviousness may justifiably follow". In re Burt, 148 U.S.P.Q. 548 (CCPA 1966).

The instantly claimed methods, compositions, and

kits are directed to enhancing the delivery of a nucleic acid molecule to a cell by providing denatured collagen or a peptide thereof to the cells in co-ordination with the nucleic acid molecule. The prior art references cited by the Examiner wholly fail to teach or suggest that denatured collagen or a peptide thereof enhances the delivery of a nucleic acid molecule. Indeed, the '902 patent expressly teaches that "the gelatin coating was necessary for cells to remain adherent during the transfections" (column 6, lines 46-47). Further, the '225 patent states that the gelatin hydrogel polymer coated on the balloon catheters allows contact with "smooth muscle cells which underlie the endothelial cell layer" (column 2, lines 56-61). Lastly, the '337 patent discloses that the gelatin contained within the microparticles is used because of its charge density and ability to complex with nucleic acids (see column 3, lines 27-29) and its ability to be linked to targeting ligands through its lysine groups (column 3, 43-47). Accordingly, the '902, '225, and '337 patents fail to teach or suggest any enhancement in the efficiency of the delivery of a nucleic acid molecule of the cell in the presence of gelatin.

Furthermore, there is considerable precedent, including cases factually analogous to the present facts, that supports Applicants' position that none of the '902, '225, and '337 patents constitute an anticipation of the claims against which they are cited. For example, in In re Marshall, 198 U.S.P.Q. 344 (C.C.P.A. 1978), the Court of Customs and Patent Appeals reversed a §102 rejection of claims directed to a weight control process using a oxethazine which was previously taught by the Physicians Desk reference (PDR) to be effective for the treatment of esophagitis, gastritis, peptic ulcer, and irritable colon syndrome. The rationale for the Court's decision was simply stated as follows:

> "Nothing in the PDR remotely suggests taking oxethazine to lose weight. If anyone ever lost weight by following the PDR teachings it was an unrecognized accident. **An**

> **accidental or unwitting duplication of an invention cannot constitute an anticipation.**" [Citation omitted; Emphasis added].

Notably, this result is consistent with the reasoning of the U.S. Supreme Court which stated in Tilghman v. Proctor, 102 U.S. 707 (1881), that if a compound was "accidentally and unwittingly produced, whilst the operators where in pursuit of other and different results, without exciting attention and without it being known what was done or how it had been done, it would be absurd to say that this was an anticipation."

More recently, a similar outcome was reached in Rappaport v. Dement, 59 U.S.P.Q.2nd 1215 (Fed. Cir. 2001), an interference in which the subject mater at issue was a method for the treatment of sleep apnea. More specifically, the interference count called for treating sleep apnea by administering a therapeutically effective amount of certain azapirone compounds, such as buspirone. The PTO Board of Appeals found that a prior publication disclosing the use of buspirone to treat anxiety in patients suffering from sleep apnea did not disclose administration of buspirone for the treatment of patients suffering from sleep apnea, per se. This determination was sustained on appeal to the Federal Circuit. The Board's decision and the Federal Circuit's affirmance thereof were based on the finding that treatment of the sleep apnea disorder itself is distinct from treatment of anxiety and other secondary symptoms related to sleep apnea. This finding was, in turn, based on the interpretation that the claim terminology "treatment of sleep apneas" should be treated as a **claim limitation**. Because the cited prior art publication failed to disclose treatment of the underlying sleep apnea disorder, as opposed to the symptoms thereof, it was held not to anticipate the count. Indeed, the Federal Circuit observed in this regard that there was no disclosure in the publication of tests in which buspirone was administered to patients suffering from sleep apnea "with the intent to cure the underlying condition." Id. at 1221.

In view of the foregoing authorities, the conclusion is inescapable that the '902, '225, and '337 patents fail to disclose the instantly claimed inventions of enhancing the efficiency of the delivery of a nucleic acid molecule to a cell in the presence of denatured collagen or a peptide thereof.

Applicants also submit that the '902, '225, and '337 patents only disclose the use of gelatin and not denatured collagen as taught in the instant application. To emphasize this distinction, Applicants have added claims 68-70 which recite that the denatured collagen or peptide thereof was denatured by boiling in acidic conditions. As taught in Example 1, collagen that is denatured under such conditions is superior to native collagen as well as collagen gelled at neutral pH at 37°C.

In view of all of the foregoing, Applicants submit that the 35 U.S.C. §102/103 rejections of Claims 34, 35, 40-46, 51-60, 65, and 66 are untenable. Withdrawal of these rejections is respectfully requested.

CONCLUSION

In view of the amendments presented herewith and the foregoing remarks, it is respectfully urged that the objections and rejections set forth in the April 21, 2006 Official Action be withdrawn and that this application be passed to issue.

In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to call the undersigned agent at the phone number given below.

Respectfully submitted,
DANN, DORFMAN, HERRELL AND SKILLMAN
A Professional Corporation

By _____
Robert C. Netter, Jr., Ph.D.
PTO Registration No. 56,422

Telephone: (215) 563-4100
Facsimile: (215) 563-4044
Enclosure: Perlstein et al., Gene Ther. (2003) 10:1420-1428

Gene Therapy (2003) 10, 1420–1428

www.nature.com/gt

DNA delivery from an intravascular stent with a denatured collagen-polylactic-polyglycolic acid-controlled release coating: mechanisms of enhanced transfection

I Perlstein[1], JM Connolly[1], X Cui[1], C Song[1], Q Li[1], PL Jones[3], Z Lu[1], S DeFelice[1], B Klugherz[2], R Wilensky[2] and RJ Levy[1]

[1]*Division of Cardiology, Children's Hospital of Philadelphia, Philadelphia, PA, USA;* [2]*Division of Cardiology, University of Pennsylvania Health System, Philadelphia, PA; and* [3]*Department of Pediatrics, University of Colorado Health Sciences Center, Section of Critical Care, Denver, CO, USA*

We previously demonstrated that DNA–polylactic–polyglycolic acid (PLGA)-coated stents can deliver genes to the arterial wall with reporter expression involving 1% of neointimal cells. The present study investigated a novel formulation utilizing denatured collagen in DNA-stent coatings; denatured collagen was hypothesized to enhance gene transfer due to adhesion molecule interactions and actin-related mechanisms. Arterial smooth muscle cells (SMCs) cultivated on denatured collagen had significantly greater plasmid DNA (β-galactosidase) transfection than SMC grown on native collagen (18.3± 1.2 vs 1.0± 0.1%, P< 0.001). The denatured-collagen effect was completely blocked with anti-$\alpha_v\beta_3$ integrin antibody. SMCs cultivated on native collagen supplemented with tenascin-C (TN-C), a protein recognized by $\alpha_v\beta_3$ integrins, showed a 33-fold increase in transfection compared to control (P< 0.001); this effect was also blocked with anti-$\alpha_v\beta_3$ antibody. We observed that cells grown on denatured collagen had marked F-actin-enriched stress fibers and intense perinuclear G actin, compared to those grown on native collagen, which demonstrated F-actin-enriched focal adhesions without perinuclear G-actin localization. Cytochalasin-D, an F actin depolymerizing agent, caused significantly increased SMC transfection in cells cultivated on native collagen compared to control cells (18.0± 1.8 vs 3.02± 0.9%, P< 0.001) further supporting the view that actin-related cytoskeletal changes influence transfection. A denatured-collagen–PLGA composite vascular stent coating similarly resulted in increased plasmid DNA green fluorescent protein (GFP) expression compared to controls (P< 0.001) in SMC cultures; the increased transfection was blocked by anti-$\alpha_v\beta_3$ antibody. Pig coronary studies comparing denatured-collagen–PLGA-coated stents containing plasmid DNA (encoding GFP) to coated stents without DNA demonstrated 10.8% of neointimal cells transfected; this level of expression was almost an order of magnitude greater than previously reported with a DNA delivery stent. It is concluded that denatured collagen incorporated into plasmid DNA-stent coating formulations may increase the level of gene expression in vitro and in vivo because of integrin-related mechanisms and associated changes in the arterial smooth muscle cell actin cytoskeleton.
Gene Therapy (2003) **10,** 1420–1428. doi:10.1038/sj.gt.3302043

Keywords: *site specific; $\alpha_v\beta_3$ integrins; actin cytoskeleton*

Introduction

Plasmid DNA delivery from intravascular stents[1] is a forefront approach for achieving gene therapy for cardiovascular disease. Controlled release of DNA from vascular stents was previously investigated by our group in a series of studies using green fluorescent protein (GFP) plasmid DNA incorporated into a polylactic–polyglycolic acid copolymer (PLGA) emulsion coating on stainless-steel stents.[1] These studies demonstrated GFP expression in cultured vascular SMCs, as well as in pig coronary arteries, with 1% of neointimal arterial smooth muscle cells transfected. Although these results demonstrated great promise, the relatively low level of transfection achieved in this initial study would likely preclude therapeutic efficacy. Therefore, the present paper focused on a mechanistic approach toward enhancing the level of transfection with DNA-delivery stents.

Prior investigations have demonstrated that specific cell adhesion receptors, including integrin heterodimers, can be used to target uptake of either plasmid DNA[2,3] or viral[4,5] vectors. However, these studies did not examine whether the addition of exogenous ligands to the extracellular matrix (ECM) affected gene expression

Correspondence: RJ Levy, Children's Hospital of Philadelphia, Abramson Research Building, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318, USA
The first two authors contributed equally
Received 29 August 2002; received in revised form 11 December 2002; accepted 12 February 2003

efficiency. Previous studies[6] have demonstrated that denatured type I collagen, but not native collagen, can upregulate Tenascin C (TN-C) in arterial smooth muscle cells (SMCs). TN-C is an ECM protein that enhances cell survival and proliferation.[7,8] Furthermore, this denatured collagen–SMC interaction can be blocked with an anti-β3 integrin antibody,[6,9] thereby indicating a specific integrin receptor interaction is responsible for this effect. In this study, we attempted to determine whether specific ECM–integrin interactions involving denatured-collagen coatings and associated actin-cytoskeletal changes would increase the extent of plasmid DNA transfection. We hypothesized that denatured-collagen coatings on a DNA-delivery stent could lead to an increased level of arterial gene expression from DNA-delivery stents.

The present study investigated DNA-controlled release using denatured collagen as a component of a DNA-delivery stent coating to hypothetically increase the level of plasmid DNA transfection through mechanisms involving β_3 integrin receptor interactions and associated changes in actin dynamics. The goals of our investigations were:

(1) To investigate the effects of a denatured-collagen substrate (an $\alpha_v\beta_3$ integrin ligand) on plasmid DNA transfection in cell culture, compared to a native type I collagen substrate
(2) To determine if the observed transfection was related to β_3 integrin interactions;
(3) To learn if TN-C, an alternative $\alpha_v\beta_3$ integrin ligand, could also positively influence transfection efficiency in cell culture
(4) To examine changes in the F and G-actin cytoskeletal distribution due to denatured collagen in culture, and to determine whether actin-specific agents affect transfection
(5) To formulate and characterize a coating that includes denatured collagen for use in a DNA-controlled release stent to deliver plasmid DNA to pig coronary arteries

Results

SMC cultivated on denatured vs native collagen demonstrate higher levels of transfection

When A10 cells were cultured on denatured collagen (Figure 1b), they demonstrated a 17-fold greater β-galactosidase transfection level then did A10 cells grown on native collagen (Figure 1a), 18.3 ± 1.2 vs $1.01\pm0.1\%$ respectively, $P<0.001$ (Figure 1e). Cell viability approached 100% (99.7 ± 0.1 vs $99.5\pm0.1\%$ respectively) on both substrates, as measured by trypan blue exclusion, and confirmed by live/dead assay of cells at 72 h (Figures 1c and d). Although there was no difference in cell adherence (12.2 ± 0.9 vs 12.3 ± 0.8 cells/mm^2 respectively at 5 h), cells cultivated on denatured collagen had a greater proliferation rate than did cells grown on native collagen, with 3.1 ± 0.7-fold more cells after 72 h compared to A10 cells cultivated on native collagen per WST-1 assays. This metabolic assay was corroborated by direct visual count of cells per mm^2 on each substrate (40.2 ± 3.2 vs 16.0 ± 0.6 cells/mm^2, respectively, in a parallel experiment). Nevertheless, the increase in transfection efficiency far exceeded the enhanced effects on growth (17.0- vs 3.1-fold).

Since denatured collagen is a known $\alpha_v\beta_3$ integrin ligand,[6,10,11] we next investigated the involvement of this receptor in modulating denatured-collagen-mediated transfection. Using integrin-function-blocking antibodies, we found that pretreating the cells with anti-$\alpha_v\beta_3$ monoclonal antibodies had no significant effect on transfection of cells cultured on native collagen ($P=0.78$, data not shown), but eliminated the enhanced transfection observed when A10 SMCs were maintained on a substrate of denatured collagen (from $18.3\pm1.2\%$ to $2.9\pm0.2\%$ $P=0.001$). Cell growth was not reduced by addition of the antibody (22.0 ± 0.1 vs 23.9 ± 1.8 cells/mm^2 in a representative 72 h experiment). Nonimmune mouse IgG had no effect on reducing the transfection efficiency ($17.9\pm0.4\%$, P=n.s.; Figure 1e), or on cell growth.

Next, we determined whether the $\alpha_v\beta_3$ integrin-dependent effects on enhanced transfection were specific for SMC interactions with denatured collagen, or whether alternate $\alpha_v\beta_3$ integrin ligands (ie TN-C) would also affect the efficiency of transfection. Addition of TN-C to a native collagen substrate resulted in a dose-dependent increase in transfection (Figure 2a). However, while the lowest dose of TNC-C used, 1-μg/ml, had no effect on transfection efficiency (Figure 2a), it resulted in a three-fold increase in proliferation per WST-1 assay (data not shown). This suggests that although TN-C also caused increased SMC proliferation in these studies, enhanced transfection and proliferation are independent events. The highest dose of TN-C used, 50-μg/ml, caused a 33-fold increase in transfection efficiency with only an associated seven-fold increase in proliferation (data not shown). Furthermore, like denatured-collagen-mediated transfection, the TN-C-mediated transfection enhancement was found to be $\alpha_v\beta_3$ dependent. Blocking antibody studies using either anti-$\alpha_v\beta_3$ or anti-β_3 or reduced transfection to levels seen without the addition of TN-C (Figure 2b).

Actin changes associated with increased transfection in vitro

Since changes in cell interactions with ECM via integrins ultimately control intracellular actin dynamics, we next investigated the relation between cellular DNA uptake and the cytoskeletal distribution of the different forms of actin. Double fluorescent label studies were performed using phalloidin (rhodamine) as a marker of F actin, and nuclease-I (FITC) as a marker of G-actin. Cells grown on native collagen were somewhat elongated and displayed prominent colocalization of F and G actins in focal adhesions in confocal images (Figure 3a). In A10 cell cultures grown on denatured collagen, we also observed a marked qualitative increase in G-actin intensity and concentration around the nucleus (Figure 3b), and prominent F-actin stress fibers, not noted in native collagen cultures.

Having shown an association between cell substrate, actin depolymerization (G actin), and transfection capacity, we sought to perform experiments to investigate a potential causal relation between manipulation of the actin cytoskeleton and transfection capacity. The F-actin

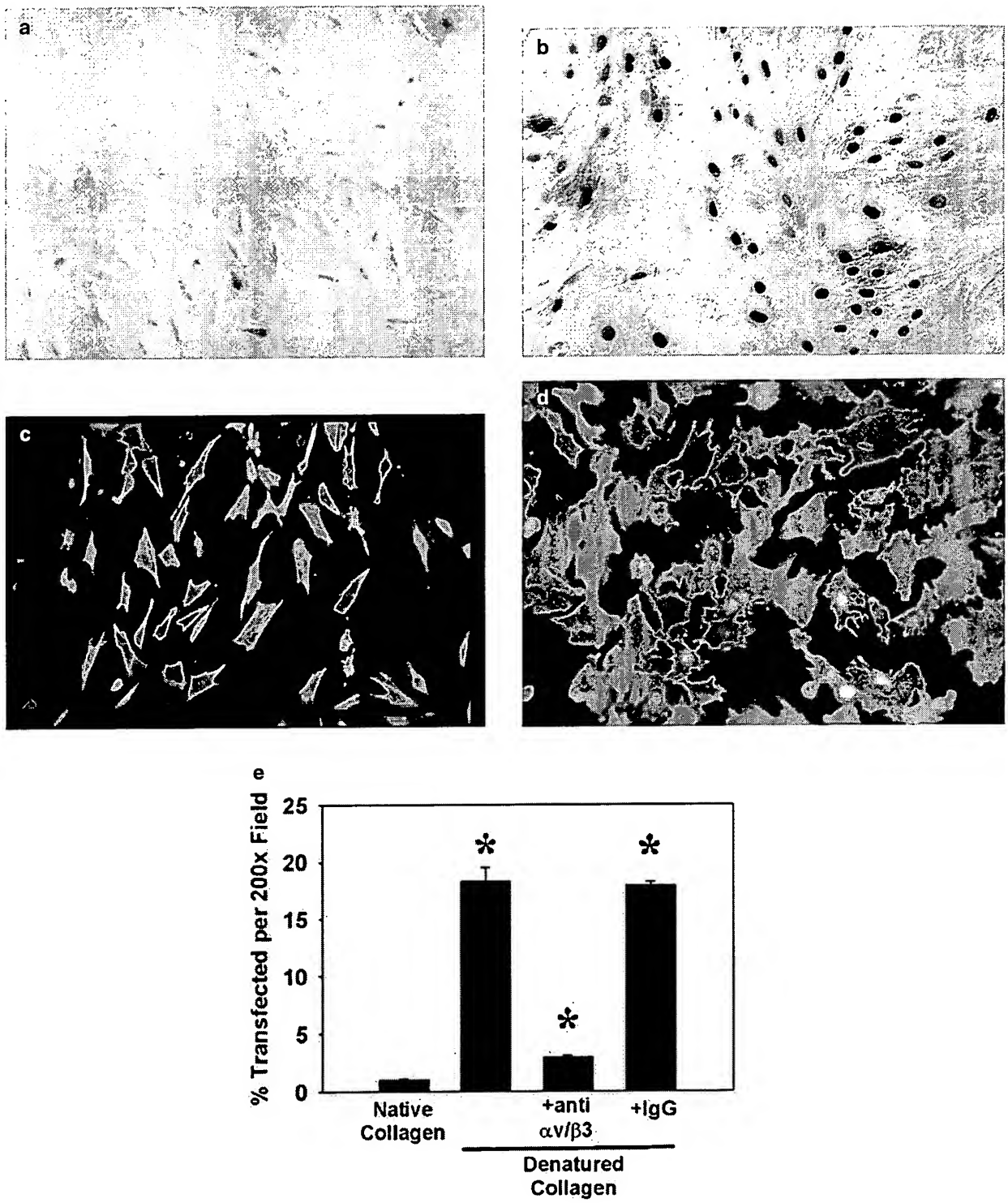

Figure 1 *Cell morphology and transfection on native or denatured collagen. (a) A10 cells grown on native collagen assume an elongated morphology and poor transfection efficiency, as indicated by the paucity of β-gal-expressing cells (dark blue). (b) Cultures grown on denatured collagen are denser, and show high transfection efficiency, indicated by the many β-gal-expressing cells. (c) A10 cells grown on native collagen (live/dead staining) with high viability per enzymatic metabolism of calcein (green fluorescence), vs retention of EthD-1 by dead cells (red fluorescence), similar to (d) A10 cells grown on denatured collagen. (e) Quantitation of cells expressing β-gal confirms significant enhancement with culture on denatured collagen, while specific antibody blockade of $\alpha_v\beta_3$ integrin cell–substrate interaction on denatured collagen reduces the transfection rate to that of cells on native collagen. Nonspecific IgG (control) had no effect on transfection. (a, b) bright-field photomicrographs of X-gal-stained cultures, (c, d) fluorescence photomicrographs of live/dead assay™ stained cultures, × 200 magnification. *$P<0.001$.*

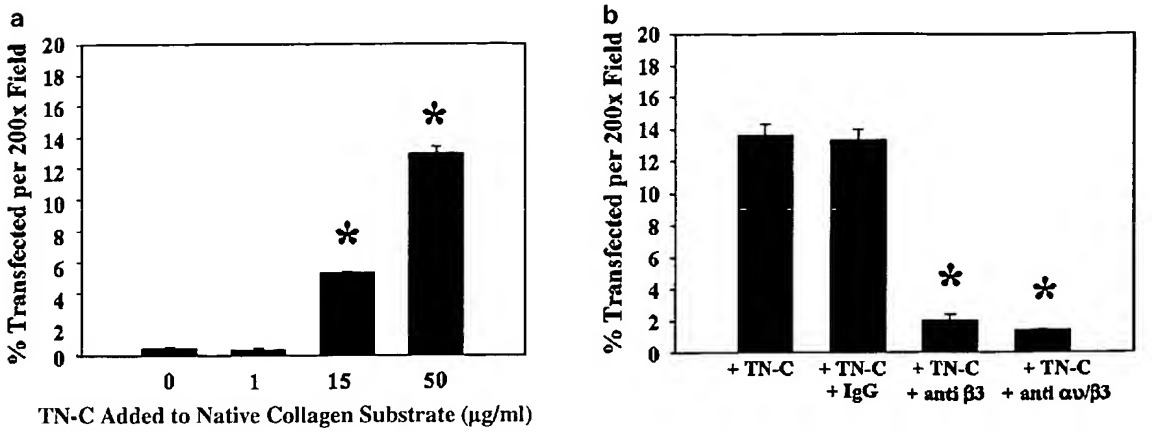

*Figure 2 Quantitation of TN-C-enhanced transfection and its dependence on $\alpha_v\beta_3$ integrin cell-native collagen interaction. (a) The addition of TN-C to a native collagen substrate causes a dose-dependent increase in transfection at concentrations at or above 15 μg/ml (*P<0.001). (b) Use of either anti-α_v/β_3 or anti-β3 antibody significantly reduces the high transfection rate seen in the presence of TN-C in native collagen (*P<0.001); nonspecific IgG did not differ from control.*

destabilizer cytochalasin-D, at a dose of 100 nM (Figure 4a), significantly enhanced transfection efficiency when compared to control cells maintained on native collagen (18.0±1.8% vs 3.02±0.9%, Figure 4b) without significantly affecting cell proliferation (30.1±3.9 vs 23.8±5.7 cells/mm^2, means±s.e., at 72 h, respectively). Moreover, the cytochalasin-D effect could be blocked via the pretreatment of the A10 cell cultures with 10 μM jasplakonilide (Figure 4b), an F-actin stabilization agent, known to counteract the F-actin depolymerization effects of cytochalasin-D (1.14±0.54%, $P<0.001$, Figure 4b). Jasplakonilide did not reduce cell proliferation (30.9±4.2 cells/mm^2).

Denatured-collagen stent-coating formulation

Having demonstrated in cultured SMCs that denatured collagen enhances the transfection efficiency of plasmid DNA, we next carried out experiments in which plasmid DNA and denatured collagen were formulated as a coating on vascular stents. It should be noted that native collagen gels, because of their fragile nature, proved to be unsuitable for stent coatings; these preparations broke up upon stent deployment with immediate shedding of the native collagen coating. Thus, no native collagen control group was possible for the *in vivo* studies.

In the present study, crown stents (Cordis, Morristown, NJ, USA) were coated with several formulations, comparing DNA dispersed in denatured collagen, to the same coating, but with an outer PLGA polymer-coating layer. These studies demonstrated (Figure 5a) that the release rate of plasmid DNA from the denatured-collagen-only coating was far too rapid for potential *in vivo* use, with virtually all of the DNA released in less than 30 min. However, successively increasing concentrations of an outer PLGA coating resulted in a more sustained release of DNA, with a release duration of more than 24 h (Figure 5a). In addition, it was necessary to crosslink the denatured-collagen coatings in order to retain these coatings as a stable robust surface layer on the vascular stents. Thus, all denatured-collagen-coated stents were treated with water-soluble carbodiimide. The plasmid DNA released from these formulations in sustained elution studies was intact, as demonstrated by agarose gels (Figure 5b).

A series of cell culture studies was carried out with vascular stents coated with the DNA-denatured collagen, plus PLGA. We observed transfection localized only to cells in culture in contact with the stent struts (data not shown), and thus confined our observations to those areas. In these experiments, we also sought to learn if the $\alpha_v\beta_3$ integrin was involved, especially since the coating formulation involved crosslinking denatured collagen followed by coating with PLGA, and thus differed markedly from the pure-denatured type I collagen cell culture substrates studied in the earlier experiments. Nevertheless, A10 cell culture blocking antibody studies using monoclonal anti-$\alpha_v\beta_3$ demonstrated significant inhibition of the $\alpha_v\beta_3$ enhanced transfection associated with the denatured-collagen–PLGA coating (Figure 5c). However, the relative magnitude of the transfection *in vitro* with denatured-collagen–PLGA stents, and its inhibition, was less than that observed with A10 cells plated directly on the collagen substrates, indicating the likely influence of other formulation steps involving the PLGA and crosslinking procedures. Nevertheless, this particular formulation demonstrated enhanced transfection in cell culture compared to controls, and furthermore, unlike native collagen, was suitable as a stent coating for intravascular studies with DNA-delivery stents.

Denatured collagen enhances DNA delivery from coronary stents in vivo

GFP expression levels in the arterial wall with denatured-collagen–PLGA-coated stents exhibited high levels of GFP fluorescence expression in the neointima (Figures 6a–c vs control artery 6d), which was confirmed by GFP immunohistochemistry (Figure 6e vs control artery 6f). Denatured-collagen–PLGA-coated stents in the present studies containing 500 μg DNA demonstrated 10.4±1.23% neointimal cells expressing GFP. Coated stents without plasmid DNA (controls) demonstrated no evidence of GFP expression (Figure 6d).

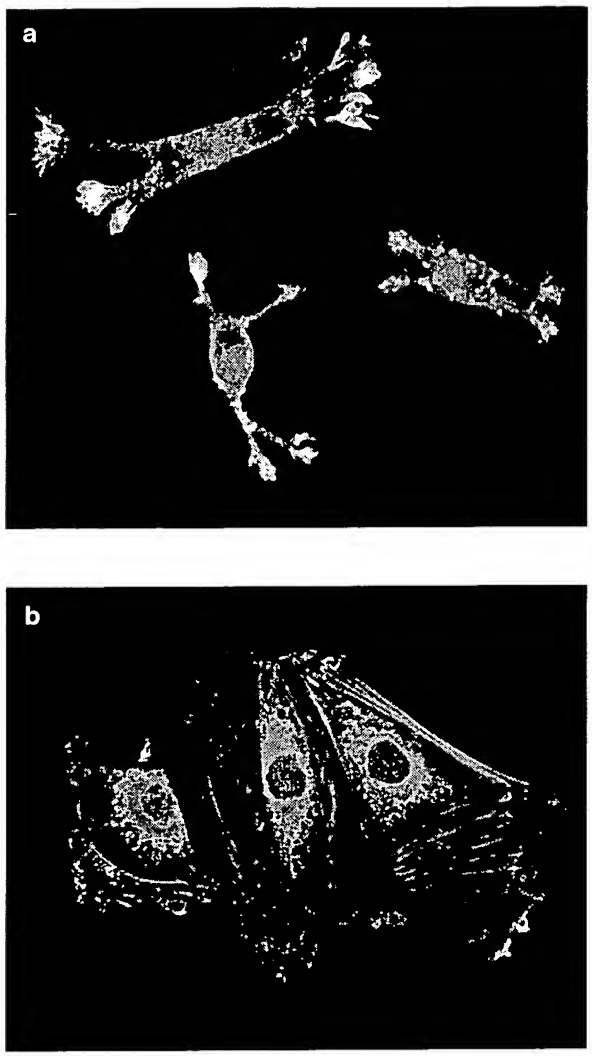

Figure 3 Changes in the arterial smooth muscle cell cytoskeletal actin distribution in A10 cells cultivated on native and denatured collagen. Double-fluorescent label studies of F and G actins were performed using phalloidin (rhodamine) as a marker of F actin, and nuclease-I (FITC) as a marker of G actin. Cells grown on native collagen (a) were relatively elongated and displayed prominent colocalization of F and G actins in focal adhesions in confocal images, while on denatured collagen (b), there was a marked qualitative increase in G-actin intensity and concentration both in the cytoplasm and around the nucleus, with prominent F-actin stress fibers at the cell perimeter. Confocal fluorescent photomicrographs, × 400.

Discussion

Our cell culture results indicate that $\alpha_v\beta_3$ integrin interactions with their cognate ligands (ie denatured collagen and TN-C) may be useful for enhancing plasmid DNA transfection. Rat arterial smooth muscle cells (A10) have been previously utilized by our group for cell culture modeling studies of gene-delivery stent functionality and mechanisms.[1,12] β_3 and $\alpha_v\beta_3$ integrins were shown by others to be upregulated in SMCs after vascular injury.[13,14] Accordingly, SMC $\alpha_v\beta_3$ integrin interactions with the ECM have been widely investigated in tissue culture and *in vivo* in order to define their role in SMC proliferation and migration mechanisms.[13,15,16] In the present study, our working hypothesis was that these same $\alpha_v\beta_3$ integrin interactions could also be involved in regulating plasmid-DNA transfection. Our integrin-blocking antibody results strongly support this (Figures 1e, 2b, 5c). Receptor signaling[9] is involved in mediating cytoskeletal changes such as those observed in the present studies. Our data also indicate that actin-cytoskeletal changes may be in part responsible for the increased transfection of SMCs cultivated on either denatured collagen or TN-C-enriched substrates compared to native collagen. We hypothesized that modification of the G-/F-actin cytoskeletal distribution might play a role in increasing transfection. G actin is an inhibitor of nuclease-I.[17] Thus, switching the cytoskeleton balance in favor of G actin or modifying the cellular actin distribution (see Figure 3b) might enhance DNA transfection based on decreased plasmid destruction.

Could the increased transfection observed in these studies be somehow related to increased proliferation observed on some of the substrates, such as denatured collagen or TN-C? The results of our cytochalasin-D experiments indicate that proliferation may not be involved in the mechanisms responsible for the observed increase in transfection (Figure 4b). In these studies, although there were no significant differences in proliferation comparing SMCs grown on native collagen to those grown on native collagen with added cytochalasin-D (see Results, above), there was significantly greater transfection (18 vs 2%) in the cytochalasin-D-treated group (Figure 4b), indicating that an actin-related mechanism may be a critical component, and that cellular proliferation-related events are occurring independently of transfection-enhancement mechanisms. This view was further confirmed through studies using the F-actin stabilizing agent, jasplakonilide, which blocked the cytochalasin-D-related increase in transfection (Figure 4b), thus providing support for an actin-related mechanism being responsible for the observed increased transfection.

A previous study by our group using a pig coronary stent angioplasty model demonstrated successful coronary artery gene transfer with a plasmid DNA delivery stent using a PLGA emulsion coating,[1] but with far less neointimal expression (1%) than observed in the present study (10.4%). These very different results arose from similar study designs: The same vector was used in both studies; comparable DNA loadings were used in both; an identical PLGA component was used in both stent formulations; both studies used Crown Stents; the animal strain, size, sex, and supplier were the same; the study durations were identical; and the operating team included all the same individuals. However, direct comparisons with our previous study[1] are not possible. Nevertheless, the increased transfection noted the present report suggests that the denatured-collagen component of the coating may have been responsible for the relatively greater transfection than that observed previously.[1] At this time, no other researchers have reported results with plasmid DNA delivery stents.

In conclusion, the present results support the view that plasmid DNA delivery from stent coatings containing $\alpha_v\beta_3$ integrin ligands, such as denatured collagen, may result in increased levels of arterial SMC transgene expression. Overall, our data indicate that this may be due to integrin-signaling mechanisms, and associated G-actin-related cytoskeletal changes.

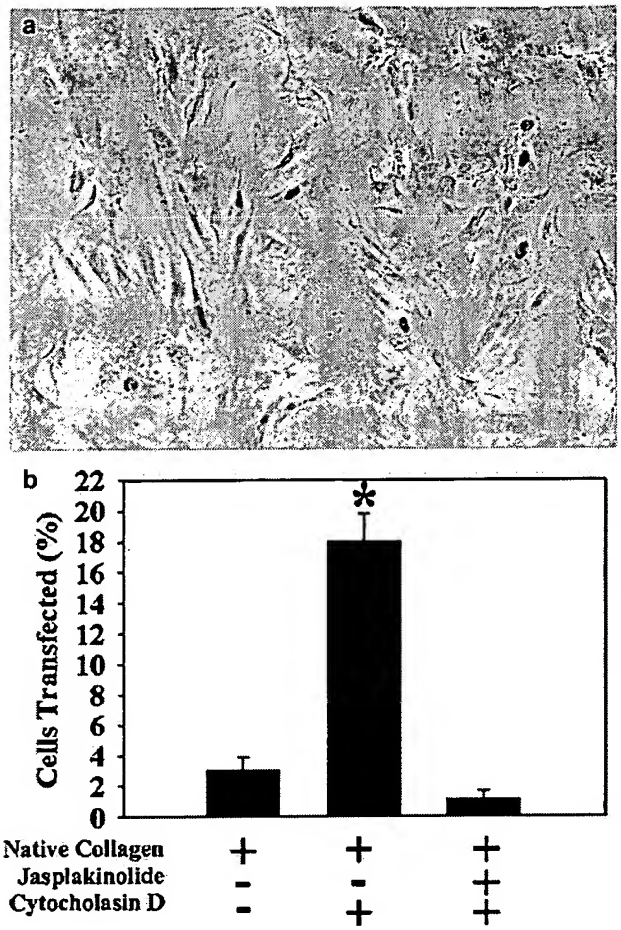

*Figure 4 Transfection and cytoskeletal manipulation. Cells grown on native collagen after treatment with the F-actin depolymerization agent, cytocholasin-D (cyto-D) (a), show increased transfection compared to a native collagen control (see in comparison Figure 1a). (a) Bright-field photomicrograph of X-gal-stained culture, ×200 magnification. (b) Quantitation of transfection, showing significant increase (*P<0.001) in transfection in the cyto-D cultures and that the blockade of the cyto-D effect with jasplakonilide results in a low transfection rate, comparable to native collagen (see also Figure 1e).*

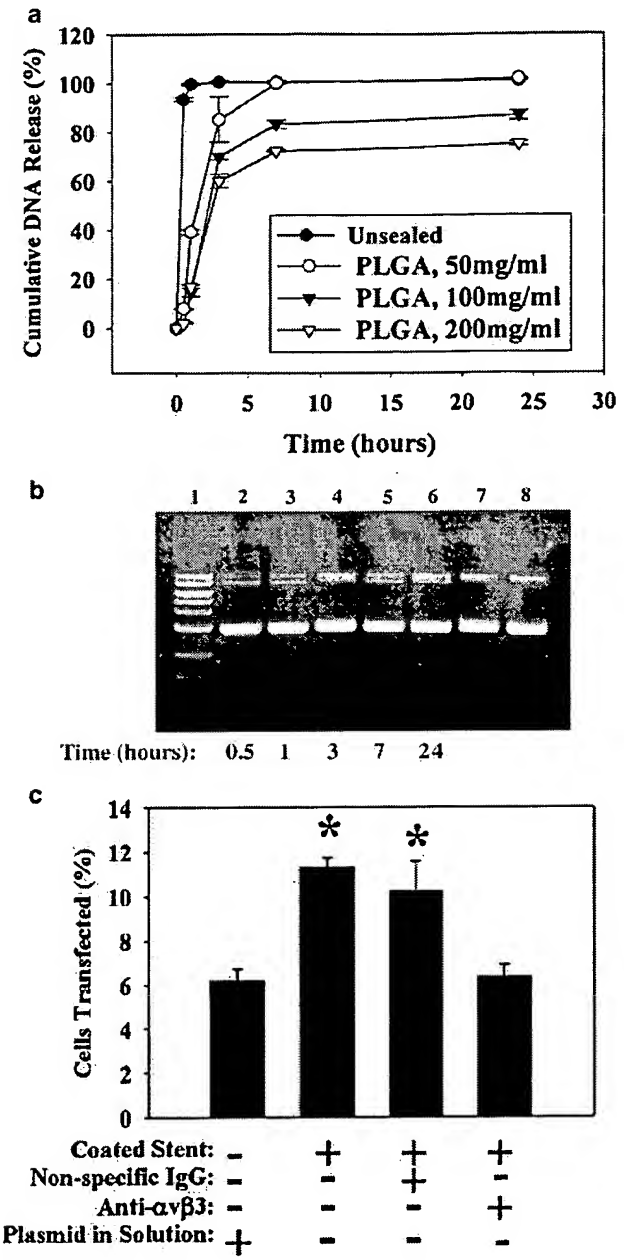

*Figure 5 Characterization of denatured-collagen-coated vascular stents. (a) Cumulative DNA release from stents coated with varying concentrations of PLGA shows controlled release of DNA load over 24 h as a function of the PLGA coating. (PLGA concentrations: closed circles=0 mg/ml; open circles=50 mg/ml; closed triangles=100 mg/ml; open triangles=200 mg/ml). (b) Agarose gel of DNA eluted from a denatured-collagen-coated vascular stent (a, 100 mg/ml PLGA condition) (lanes 1–6) shows the integrity of delivered DNA compared to control plasmid stock over time (lanes 7 and 8). Lane 1; 1 kb DNA ladder. (c) Transfection of A10 cells in vitro by contact with coated stents is dependent on stent coating and cell-$\alpha_v\beta_3$-integrin contact, as demonstrated with anti-integrin antibodies. *P<0.001.*

Materials and methods

Collagen preparation

Native collagen: Type I collagen (3.1 mg/ml, Vitronectin 100, Cohesion Technologies, Palo Alto, CA, USA) was neutralized with 0.1 N NaOH and 10×PBS to a concentration of 2.5 mg/ml, and gelled at 37°C according to the manufacturer's directions. Denatured Collagen: Type I collagen was acidified with 0.17% glacial acetic acid (v/v), boiled for 1 h, then neutralized as above. Denatured collagen was allowed to air-dry on the desired surface. In experiments testing the effect of TN-C (Gibco) on transfection, TN-C was mixed into the native collagen solution at the indicated concentrations prior to well coating, and gelled at 37°C.

Transfection in vitro

A fetal rat arterial SMC line (A10, American Type Culture Collection, Rockville, MD, USA) utilized in previous studies modeling gene-delivery stents[1,12] was used for all tissue culture experiments, and was routinely maintained in growth medium consisting of M199 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (PS, Gibco). A10

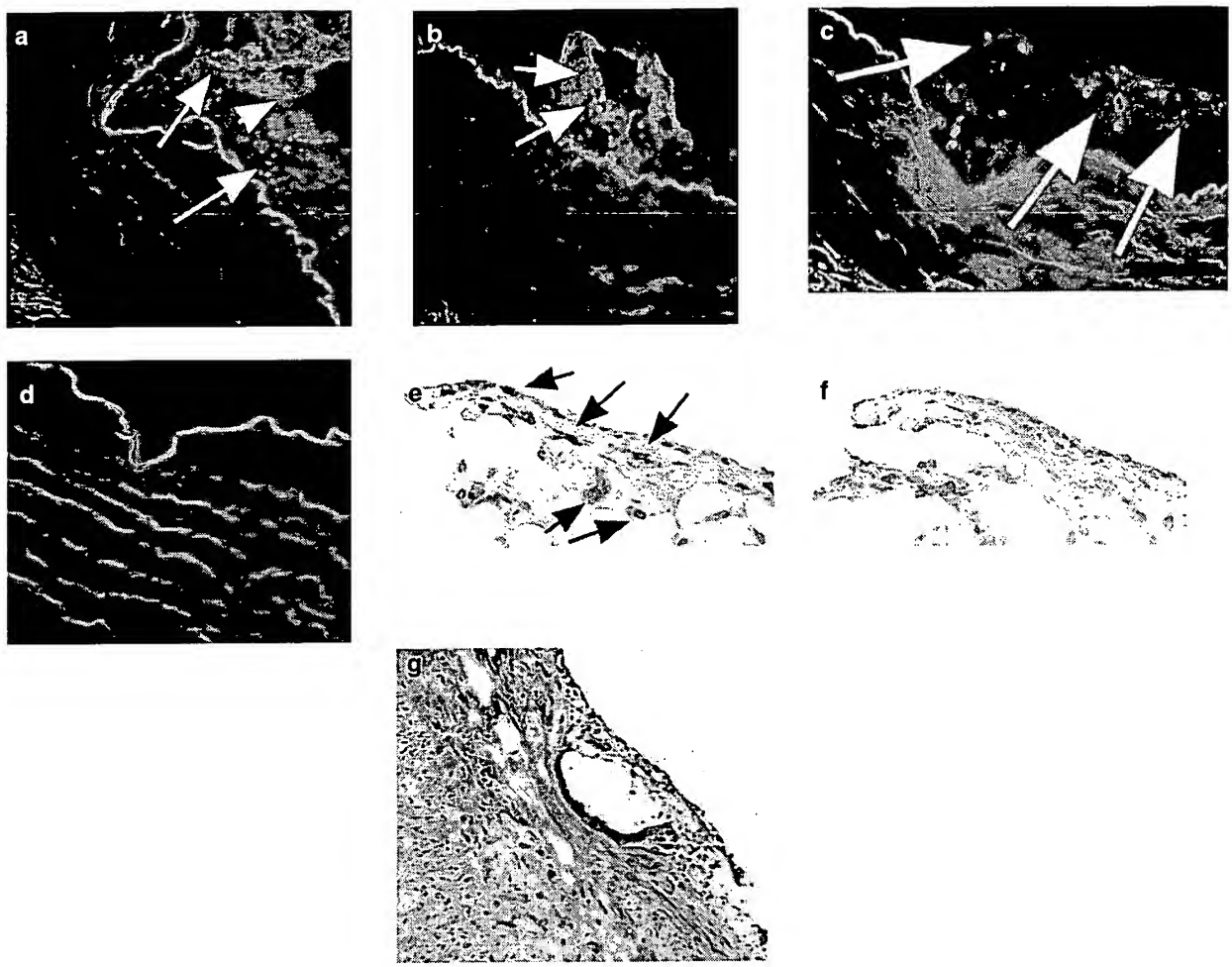

Figure 6 Plasmid delivery in vivo *using denatured-collagen-coated vascular stents. (a–c) FITC-fluorescent micrographs showing examples of GFP reporter gene expression 7 days after stent deployment, with enhanced neointimal GFP (arrows) expression. GFP expression is absent in arteries stented without GFP plasmid (d). Serial sections of neointima shown in (c) are the subject of confirmatory GFP (arrows) immunohistochemistry shown in (e), in which purple (vector VIP, see Materials and Methods) denotes GFP-positive cells, and negative control immunohistochemistry (nonimmune IgG), shown in (f). (g) Hematoxylin and eosin of stented artery showing early neointimal formation prominent around the site of a stent post and inflammation of the stented arterial media (original magnifications* × 200).

cells were plated at 1×10^5 cells/well in six-well plates 20 h in advance of transfection. Transfection of A10 cells was performed using plasmid DNA and Lipofectamine (Gibco) according to the manufacturer's directions. Typical formulations used 2.5 μg plasmid DNA (β-galactosidase, pNGVL1-nt-β-gal, University of Michigan, Ann Arbor, MI, USA), with 5 μl Lipofectamine. Cells were fixed at 72 h using 4% paraformaldehyde.

Integrin blocking

A10 cells were pretreated with either mouse monoclonal anti-rat β_3 integrin IgG (25 μg/ml, CD61*, PharMingin, San Diego, CA, USA), mouse monoclonal anti-human $\alpha_v\beta_3$ integrin IgG (15 μg/ml, LM609, Chemicon, Temecula, CA, USA), or with equivalent concentration of nonimmune mouse IgG* (Pierce, Rockford, IL, USA) at the time of plating as previously described[9] (*sodium azide was removed by microdialysis (Slide-A-Lyzer, MWCO 10 kDa, Pierce, Rockford, IL, USA)). Cells were suspended at 1×10^5 cells/ml in growth medium containing the antibody, and then plated. Incubations and transfection then proceeded in the same manner as for nontreated cells.

β-Galactosidase expression

Medium was aspirated from cultures, which were then fixed with 4% paraformaldehyde. This in turn was rinsed twice from the cultures with 2 mM $MgCl_2$ in PBS, and replaced with X-gal stain.[18] Cultures were incubated overnight at 37°C, and then washed three times with PBS. The total number of cells and the number staining positive for X-gal were visually counted per × 100 field, and the per cent positive per well determined from the mean of 10 fields per well.

Cell attachment, proliferation, and viability

Triplicate cultures plated under conditions as described were incubated in six-well plates for 5 h at 37°C prior to visual counting of cells attached in at least three × 100 fields (10.7 mm^2) per well for quantification of attachment. Cell number per mm^2 was again determined in this manner at 72 h after treatments as described. Cell

growth was also quantified in parallel 96-well plates using a commercially available kit based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases in viable cells (WST-1, Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's directions. Trypan blue exclusion and cell count per field were performed in parallel cultures to determine culture viability, and confirmed using a Live/Dead Assay™ (Molecular Probes, Eugene, OR, USA), which is based on the ability of viable cells to enzymatically convert calcein AM to fluorescent calcein, as per the manufacturer's directions.

Double-fluorescent labeling

Cells were fixed with 4% paraformaldeyde at 6 h. Cultures were then stained for either F or G actin (Rhodamine 514 phalloidin, or Oregon green 488 Deoxyribonuclease I, respectively, Molecular Probes), following permeabilization and blocking, according to the manufacturer's directions. Cultures were then mounted using Vectashield mounting medium containing 4,6-diamidino-2-phenylindole mount (DAPI, Vector Labs, Burlingame, CA, USA) for visualization of nuclei, and confocal images recorded using a Leica TCS4D confocal microscope at $\times 400$ magnification.

Cytoskeletal manipulation

Cells were allowed to adhere to native collagen-coated six-well plates as above, then growth medium was replaced by serum-free M199, and transfection with plasmid allowed to proceed for 3–5 h. The medium was changed again to M199/2% FBS containing either 10 μM Jasplakinolide (jas, Molecular Probes) or 0.7% methanol (vehicle). Cells were incubated for 30 min and medium again replaced with M199, without or with 100 nM cytocholasin-D (cytoD, Sigma, St Louis, MO, USA) for an additional 30 min. After another medium change to remove jas or cytoD, incubations proceeded in the same manner as for nontreated cells, in M199/2% FBS, and transfection evaluated at 72 h. In order to evaluate the effect of cytoskeletal manipulation with jas and cytoD on the growth of the cells on collagen, cells were plated as above on native collagen and treated with cytoD or jas as above. After the treatments, the medium was replaced with growth medium for 72 h and assayed with WST-1 as above.

Stent-coating formulation

Denatured collagen was mixed with 10% (w/w) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, St Louis, MO, USA) as a crosslinking agent to achieve a robust coating. This mixture was incubated at 37°C for 30 min prior to use as a stent coating. GFP Plasmid DNA (pEGFP-N3, Clontech, Palo Alto, CA, USA) was added to the above collagen solution and stored at 4°C. The formulation was such that fully coated stents carried 500 μg DNA. The mixture was applied in 10 μl increments under sterile conditions onto the stent surface of 15 mm Crown stents (Cordis, Warren, NJ, USA), with manual rotation, to form a thin, uniform coating, and dried at 37°C under vacuum. The procedure was repeated until a total of 400 μl (1 mg of collagen) was coated per stent. Coated stents were dipped briefly in 100 mg/ml PLGA (MW 50 000, Birmingham Polymers, Birmingham, AL, USA) and air-dried. This thin coating was shown to be optimal for preventing rapid DNA release by preliminary experiments in which *in vitro* DNA release from stents coated with designated concentrations of PLGA was characterized.

Collagen-plasmid DNA-coated stents were shaken in 100 μl TE buffer (pH 7.4) at 37°C. Buffer was removed and replaced with fresh buffer after 0.5, 1, 2, 3, 7, and 24 h of shaking. The DNA concentrations in the collected buffers were determined by UV spectrometry (GeneQuantPro, Amersham Pharmacia Biotech), and checked for structural integrity by running 0.3 μg/lane on an agarose gel in comparison to untreated plasmid.

Transfection via stent in vitro

A10 SMCs were prepared in uncoated six-well plates as described above, prior to transfection. Coated stents were incubated in 300 μl serum-free medium at 37°C for 20–30 min with the addition of 5 μl Lipofectin (GIBCO) for 15 min. After aspiration of medium from the cell cultures, stents and their incubation medium, diluted to 1 ml, were transferred to the cell cultures and incubated at 37°C for 5 h. FBS was then added to a final concentration of 5%, and changed to growth medium after 24 h. After fixation, cultures were DAPI mounted for visualization of nuclei, and total cell number determined per $\times 200$ field in immediate contact with the stent. GFP-expressing cells were also visually counted in the same fields, and results reported as the percentage of cells transfected (mean$\pm$s.e.) of at least five fields per culture in replicate cultures.

Transfection via stent: in vivo

Animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. Denatured-collagen–PLGA-coated stents (either with GFP-plasmid loading or control without vector), prepared as described above, were deployed using two stents per animal in both the left ascending or left circumflex coronary arteries (LAD, LCX) of seven normal Yorkshire swine as previously described by our group.[1] Animals were euthanized after 7 days and representative samples of stented arteries, arterial segments distal to the stents, and unstented control pig coronary arteries were prepared for morphology studies by first removing the stent from the arteries and then embedding in frozen section media (O.C.T., Tissue-Tek, Torrance, CA, USA). The percentage of GFP-positive cells, normalizing for DAPI-positive cells, in representative frozen sections of each of the 14 stented arteries and nonstented arteries was determined according to previously published methodology, separately reporting neointimal and medial expression.[1,12] GFP expression was confirmed with anti-GFP immunohistochemistry,[1,12] and hematoxylin and eosin sections were prepared for routine light microscopy.

Statistical analysis

Data for all experiments were expressed as means plus or minus the standard error of the mean (s.e.). The significance of differences was assessed using Student's *t*-test, and was termed significant when $P \leq 0.05$.

Acknowledgements

We thank Ms Jennifer LeBold for her assistance in preparing this manuscript. This research was supported by NIH Grant HL-72108, an American Heart Association Fellowship (IP), and the William J Rashkind Endowment of the Children's Hospital of Philadelphia.

References

1 Klugherz BD *et al*. Gene delivery from a DNA controlled-release stent in porcine coronary arteries. *Nat Biotechnol* 2000; **18**: 1181–1184.
2 Collins L *et al*. *In vitro* investigation of factors important for the delivery of an integrin-targeted nonviral DNA vector in organ transplantation. *Transplantation* 2000; **69**: 1168–1176.
3 Jenkins RG *et al*. An integrin-targeted non-viral vector for pulmonary gene therapy. *Gene Therapy* 2000; **7**: 393–400.
4 Kibbe MR *et al*. Optimizing cardiovascular gene therapy: increased vascular gene transfer with modified adenoviral vectors. *Arch Surg* 2000; **135**: 191–197.
5 Wickham TJ, Carrion ME, Kovesdi I. Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. *Gene Therapy* 1995; **2**: 750–756.
6 Jones PL, Jones FS, Zhou B, Rabinovitch M. Induction of vascular smooth muscle cell tenascin-C gene expression by denatured type I collagen is dependent upon a beta3 integrin-mediated mitogen-activated protein kinase pathway and a 122-base pair promoter element. *J Cell Sci* 1999; **112**: 435–445.
7 Jones PL, Jones FS. Tenascin-C in development and disease: gene regulation and cell function. *Matrix Biol* 2000; **19**: 581–596.
8 Jones FS, Jones PL. The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Dev Dyn* 2000; **218**: 235–259.
9 Jones PL, Crack J, Rabinovitch M. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol* 1997; **139**: 279–293.
10 Montgomery AM, Reisfeld RA, Cheresh DA. Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc Natl Acad Sci USA* 1994; **91**: 8856–8860.
11 Yamamoto M, Yamato M, Aoyagi M, Yamamoto K. Identification of integrins involved in cell adhesion to native and denatured type I collagens and the phenotypic transition of rabbit arterial smooth muscle cells. *Exp Cell Res* 1995; **219**: 249–256.
12 Klugherz BD *et al*. Gene delivery to pig coronary arteries from stents carrying antibody-tethered adenovirus. *Hum Gene Ther* 2002; **13**: 443–454.
13 Stouffer GA *et al*. Beta3 integrins are upregulated after vascular injury and modulate thrombospondin- and thrombin-induced proliferation of cultured smooth muscle cells. *Circulation* 1998; **97**: 907–915.
14 Srivatsa SS *et al*. Selective alpha v beta 3 integrin blockade potently limits neointimal hyperplasia and lumen stenosis following deep coronary arterial stent injury: evidence for the functional importance of integrin alpha v beta 3 and osteopontin expression during neointima formation. *Cardiovasc Res* 1997; **36**: 408–428.
15 Brown SL, Lundgren CH, Nordt T, Fujii S. Stimulation of migration of human aortic smooth muscle cells by vitronectin: implications for atherosclerosis. *Cardiovasc Res* 1994; **28**: 1815–1820.
16 Liaw L *et al*. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin *in vitro*. *J Clin Invest* 1995; **95**: 713–724.
17 Lazarides E, Lindberg U. Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proc Natl Acad Sci USA* 1974; **71**: 4742–4746.
18 Levy RJ *et al*. Localized adenovirus gene delivery using antiviral IgG complexation. *Gene Therapy* 2001; **8**: 659–667.